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In-vitro Evaluation of Biphenylyl Acetic Acid-β-Cyclodextrin Conjugates as Colon-targeting Prodrugs: Drug Release Behaviour in Rat Biological Media

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Abstract

Biphenylyl acetic acid was selectively conjugated to one of the primary hydroxyl groups of β-cyclodextrin through an ester- or amide-linkage, and the physicochemical properties (aqueous solubility and hydrolysis) were investigated.

Aqueous solubility of the conjugates was lower than those of either drug or parent β-cyclodextrin. The amide conjugate was stable in aqueous solution and in rat biological fluids and gastrointestinal contents. The ester conjugate was hydrolysed to β-cyclodextrin and biphenylyl acetic acid at moderate rates resulting in a V-shaped rate–pH profile in aqueous solution. The ester conjugate released the drug preferentially when incubated with the contents of caecum or colon, whereas no appreciable drug release was observed on incubation with contents of stomach or intestine, nor on incubation with intestinal or liver homogenates, nor on incubation with rat blood.

The present results suggest that the ester-type drug conjugate of β-cyclodextrin may serve as a colon-targeting prodrug.

Cyclodextrins (CyDs) are known to form inclusion complexes with various drug molecules where the complex is in an equilibrium with guest and host molecules in aqueous solution (Bender & Komiyama 1978; Saenger 1980). This unique property offers a desirable quality as a drug carrier, because the complex dissociates to free CyDs and drug in absorption sites and only the drug in free form enters into the systemic circulation (Uekama & Otagiri 1978; Szejtli 1988; Duchêne 1991). The dissociation of the CyD complex is further facilitated by the competitive inclusion with hydrophobic biological components such as lipids and cholesterol in blood, thus giving little change in pharmacokinetic and pharmacodynamic behaviour of the drug after intravenous injection of the complex (Uekama et al 1981; Arimori & Uekama 1987).

On the other hand, physico- and bio-pharmaceutical properties of the CyD conjugate in which a drug is covalently bound to CyDs, may differ greatly from those of the inclusion complex. CyDs are known to be absorbed only in small quantities from the small intestine whereas they are fermented in colon microflora with a different rate depending on the cavity size, and absorbed as small saccharides from the gastrointestinal tract (Andersen et al 1983; Gerloczy et al 1985; Suzuki & Sato 1985; Flourie et al 1993). This property of CyDs may be particularly useful as a colon-targeting carrier (Friend 1992) and thus CyD conjugates may serve as a source of colon-targeting prodrugs. Although several CyD-drug conjugates have been prepared (Djedaini-Pilard et al 1993; Karunaratne et al 1993; Tanaka et al 1994) and the enantioselective hydrolysis described (Coates et al 1994), no information is available about the pharmaceutical properties of CyD conjugates. In this study, we prepared two CyD conjugates (Fig. 1) where one primary hydroxyl group of β-CyD is substituted by biphenylyl acetic acid (BPAA), an anti-inflammatory drug, through an ester- or amide-linkage, and the physicochemical properties and the drug-release behaviour in rat biological fluids and gastrointestinal contents were investigated. BPAA was chosen as a test drug for β-CyD conjugates, because of the convenience of the selective substitution of one of the β-CyD hydroxyl groups by a carboxyl group of the drug.

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Materials and Methods

Materials

β-CyD was donated by Nihon Shokuhin Kako Co. (Tokyo, Japan). BPAA and its sodium salt were supplied from Lederle Japan Co. Ltd. (Saitama, Japan). *p*-Toluenesulphonyl chloride, sodium azide, triphenylphosphine, hydroxysuccinimide and dicyclohexycarbodiimide (DCC) were purchased from Nakarai Tesq Co. (Tokyo, Japan). All other chemicals and solvents were of analytical reagent grade, and de-ionized double-distilled water was used throughout the study.

Preparation of conjugates

6^A-Deoxy-6^A-amino{(4-biphenylyl)-acetyl}-β-CyD (amide conjugate) was prepared according to the method of Bellanger & Perly (1992). To BPAA (38 mg)/anhydrous ethyl acetate (5 mL) solution was added hydroxysuccinimide (20 mg) and DCC (37 mg) and the mixture was stirred at room temperature (25°C) for 4 h. The resulting precipitates were filtered and the filtrate was dried under reduced pressure. 6^A-Deoxy-6^A-amino-β-CyD (200 mg) was added to the residue (activated BPAA ester)/dimethylformamide (DMF, 5 mL) and the mixture was stirred at room temperature for 5 h. The reaction solution was

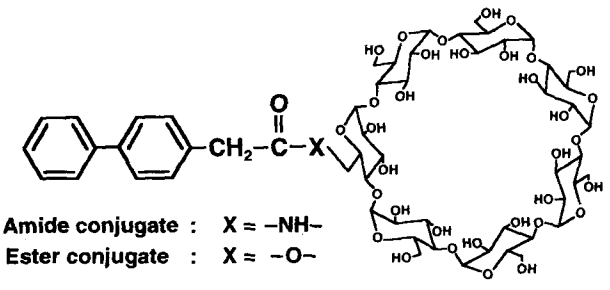

FIG. 1. Structure of BPAA-β-CyD conjugates.

concentrated under reduced pressure, and acetone (300 mL) was added. The resulting precipitates were filtered and purified by an ion-exchange column chromatography (Amberlite IR-120B) using an eluant (DMF/water 2 : 1 v/v). 6^A-Deoxy-6^A-amino-β-CyD was prepared according to the method of Hamasaki et al (1993), i.e. selective substitution of one primary hydroxyl group of β-CyD by *p*-toluenesulphonyl chloride, followed by treatment with sodium azide and reduction by triphenylphosphine.

6^A-*O*-{(4-Biphenylyl)-acetyl}-β-CyD (ester conjugate) was prepared according to the method of Coates et al (1994). Sodium 4-biphenylyl acetate (2 g) was added to 6^A-*O*-(*p*-toluenesulphonyl)-β-CyD (12 g)/DMF (100 mL), and the mixture was stirred at 100°C for 30 h. The reaction solution was concentrated under reduced pressure, and a large amount of acetone was added. The resulting precipitates were filtered and then redissolved in a small amount of DMF, to which a large amount of acetone or water was added to obtain the ester conjugate. This procedure was repeated several times to remove the remaining parent β-CyD. The sample was further purified by preparative thin-layer chromatography (Silica gel 60F (Merck F_{265}), eluant: acetonitrile/water 3 : 7 v/v). The chemical structure of the conjugates was determined by nuclear magnetic resonance, mass spectroscopy (NMR, MS) and elementary analysis, which will be reported elsewhere.

Solubility measurement of conjugates

Excess amounts (about 2 mg) of the amide and ester conjugates were added to water (1.0 mL) and the mixtures were shaken at 25°C for about 2 weeks for the former or for 1 day for the latter. The sample solution was filtered through a Dismic 3CP filter (Advantec, Tokyo, Japan), and the filtrate was analysed for the conjugates by high-performance liquid chromatography (HPLC) under the following conditions: a 655A-11 pump (Hitachi, Tokyo, Japan), an ODS-1161 column (3·0 μm, 6 mm diam. × 100 mm length, ERC, Tokyo, Japan), methanol/0·1 M acetic acid (1 : 1 v/v) as a mobile phase, a flow rate of 1·0 mL min^{-1}, and a L-4000 UV monitor (255 nm, Hitachi, Tokyo, Japan). No degradation of the conjugates was observed under these experimental conditions.

Hydrolysis in aqueous solution

The hydrolysis was performed at an initial concentration of the conjugates ($1·0 \times 10^{-6}$ M) in 0·1 v/v DMF/NaOH (0·1–2·0 M) solutions at 60°C for the amide conjugate or in 0·1 v/v DMF/phosphate or KCl–HCl buffers (pH 1·2–11·0) at 37°C for the ester conjugate. At timed intervals, an aliquot (200 μL) was neutralized by the addition of HCl (0·2–4·0 M) or NaOH (0·1 M) and analysed for the conjugates by HPLC under the same conditions described above for the solubility measurement.

Hydrolysis in rat gastrointestinal tract contents

Male Wistar rats, 400–500 g, were used for hydrolysis studies with gastrointestinal tract contents and biological media and were fed a standard diet (CE-2, CLEA Japan Inc., Tokyo, Japan). The rats were killed by decapitation and the stomach, proximal small intestine, distal small intestine, caecum and colon were removed and the contents were diluted generally to 20 w/v with chilled isotonic buffer. Stomach contents were diluted with acetate buffer (pH 4·4) whereas phosphate buffer (pH 7.4) was used for other contents, and the dispersion of contents was filtered through a gauze to remove large particulates. The conjugate solution (4·0 mL $2·0 \times 10^{-5}$ M in 2·0 v/v DMF/pH 7·4 isotonic phosphate buffer or /pH 4·4 acetate buffer) was added to the filtrate (4·0 mL) at 37°C. Thus, the initial concentrations of conjugates and DMF were $1·0 \times 10^{-5}$ M and 1·0 v/v, respectively, and the incubation solution contained 10 w/v gastrointestinal contents except for the maximal concentration (6·7 w/v) of caecal contents. The pH of incubation solutions was adjusted to 4·4 (stomach) or 7·4 (other contents) by the addition of small amounts of 0·5 M NaOH. Anaerobic conditions were simulated by using air-tight vessels and flushing the reaction suspension with nitrogen gas for 30 min before the incubation. At appropriate intervals, an aliquot (0·5 mL) of the reaction solution was added to 0·1 M HCl (0·2 mL) and BPAA was extracted with cyclohexane/ethyl ether (3 : 1 v/v, 6·0 mL) containing an internal standard, flurbiprofen (0·5 mL of 0·1–1·0 μg mL^{-1}). The organic phase (5·0 mL) was evaporated under reduced pressure and redissolved in methanol (100 μL), 20 μL of which was subjected to HPLC for determination of BPAA under the same conditions described above for the kinetic experiments in aqueous solution, except that the mobile phase was methanol/0·1 M acetic acid (7 : 3 v/v).

Hydrolysis in rat biological media

Blood. Rat blood was collected using injection syringes treated with sodium citrate. The conjugate solution (5·0 mL $2·0 \times 10^{-5}$ M in 2·0 v/v DMF/pH 7·4 isotonic phosphate buffer) was added to 5·0 mL blood at 37°C. The sampling and analysis procedures were as those described above.

Intestine homogenates. Rat intestine segments without contents were removed, cut into small pieces and homogenized with 5 vol cold 1·15 w/v KCl using a tissue homogenizer (Physcotron NS-50, Nichion, Tokyo) at 0°C. The homogenates were filtered through gauze. The conjugate solution (2·0 mL of $2·0 \times 10^{-5}$ M in 2·0 v/v DMF/pH 7·4 isotonic phosphate buffer) was added to the filtrate (2·0 mL) at 37°C. The sampling and analysis procedures were as described above.

Liver homogenates. Rat liver (wet weight about 20 g) was washed thoroughly with saline (about 500 mL), and homogenized with 5 vol cold 1·15 w/v KCl using a tissue homogenizer (Potter-Elvehjem, Corning Glass, USA) at

Table 1. Some physical properties of BPAA, β-CyD and conjugates.

Property	BPAA	β-CyD	Amide conjugate	Ester conjugate
Molecular weight	212	1135	1328	1329
Melting point (°C)	164–165	280	258–263[a]	258–268[a]
Description	white crystal	white crystal	white powder	white powder
Solubility (M)[b]	1.26×10^{-4}	1.63×10^{-2}	2.39×10^{-6}	$< 1.31 \times 10^{-5}$[c]
$[\alpha]_D^d$	+0	+125	+111	+112

[a]Decomposition. [b]In water at 25°C. [c]Could not be determined accurately because of the hydrolysis. [d]Specific rotation in DMF at 25°C.

0°C. The homogenates were filtered through gauze, and the filtrate was centrifuged at 9000*g* for 30 min at 4°C. The supernatant (2·0 mL) was added to the conjugate solution (8·0 mL of 1.25×10^{-5} M in 1·25 v/v DMF/pH 7·4 isotonic phosphate buffer) containing $MgCl_2$ 101·6 mg mL^{-1}, glucose 6-phosphate 60·8 mg mL^{-1}, nicotinamide 9·16 mg mL^{-1} and nicotinamide-adenine dinucleotide phosphate 3·35 mg mL^{-1} at 37°C. The sampling and analysis procedures were as described above.

Results and Discussion

Table 1 shows some physical properties of the conjugates. Both conjugates were confirmed, by means of NMR, MS, to be a single component, in which BPAA was introduced to one of the primary hydroxyl groups of β-CyD through an amide or ester linkage. The conjugates were obtained as white powder and were only very slightly soluble in water. BPAA was reported to be solubilized by the inclusion complexation with CyDs (Arima et al 1990). However, the solubility of conjugates unexpectedly decreased far below that of the drug. This may be due to a blockage in the protolytic dissociation of the BPAA carboxyl group through the derivatization. In addition, the low solubility of the conjugates may be at least partly ascribed to the strong intermolecular association between the biphenylyl moiety and the neighbouring CyD cavity in the solid state, forming a stable columnar packing structure, as reported for the monosubstituted CyDs (Harata et al 1993).

The hydrolysis behaviour of the conjugates in aqueous solution and in rat biological fluids and contents was investigated for its colon-targeting property as a novel drug carrier. Fig. 2 shows the reaction profiles for the hydrolysis of the amide conjugate in 0·1, 1·0 and 2·0 M NaOH solution at 60°C, where the hydrolysis proceeded according to first-order kinetics and the conjugate released BPAA quantitatively. It is apparent that the amide conjugate was fairly stable in aqueous solution; for example, the half-life in 2·0 M NaOH at 60°C was 8·6 h and no hydrolysis took place in 0·1 M NaOH at 60°C for 12 h under the experimental conditions. On the other hand, the ester conjugate was hydrolysed at moderate rates at 37°C, as is apparent from the hydrolysis rate–pH profile (Fig. 3). The pH-profile exhibited a V-shaped curve with slopes of unity and −0·6 in alkaline and acidic regions, respectively, suggesting involvements of a specific-base catalysis in the alkaline region and specific- and general-acid catalysis in the acidic region (Frost & Pearson 1961). The half-lives of the ester conjugate at pH 1·2 and pH 7·4 at 37°C were about 6·5 h and 10 days, respectively.

Fig. 4 shows the release profiles of BPAA from the two conjugates in rat gastrointestinal contents. In the contents of stomach and proximal and distal small intestines and in intestinal homogenates, the ester conjugate released the drug only in small amounts (<10%) which could be accounted for by spontaneous hydrolysis in aqueous solution. From the amide conjugate, no release of the drug took place for 48 h under the experimental conditions. On the

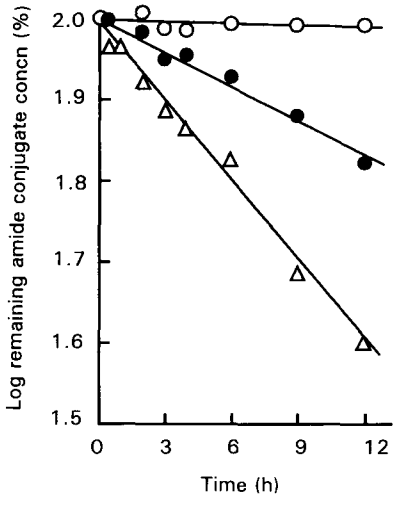

FIG. 2. Semi-logarithmic plots for hydrolysis of amide conjugate (1.0×10^{-6} M) in aqueous NaOH/DMF (0·1 v/v) solutions at 60°C. ○ 0·1 M NaOH, ● 1·0 M NaOH, △ 2·0 M NaOH.

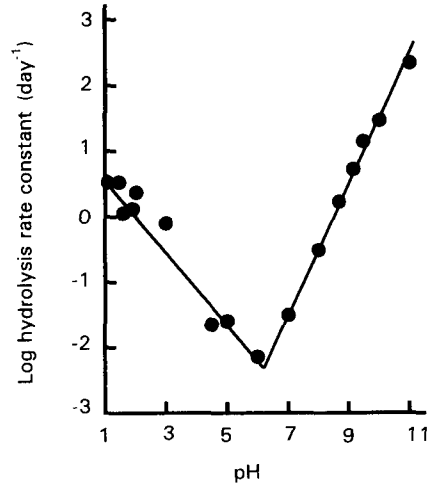

FIG. 3. pH-Profile for hydrolysis rate of ester conjugate (1.0×10^{-6} M) in phosphate or KCl/HCl buffer (μ = 0·2)/DMF (0·1 v/v) solutions at 37°C.

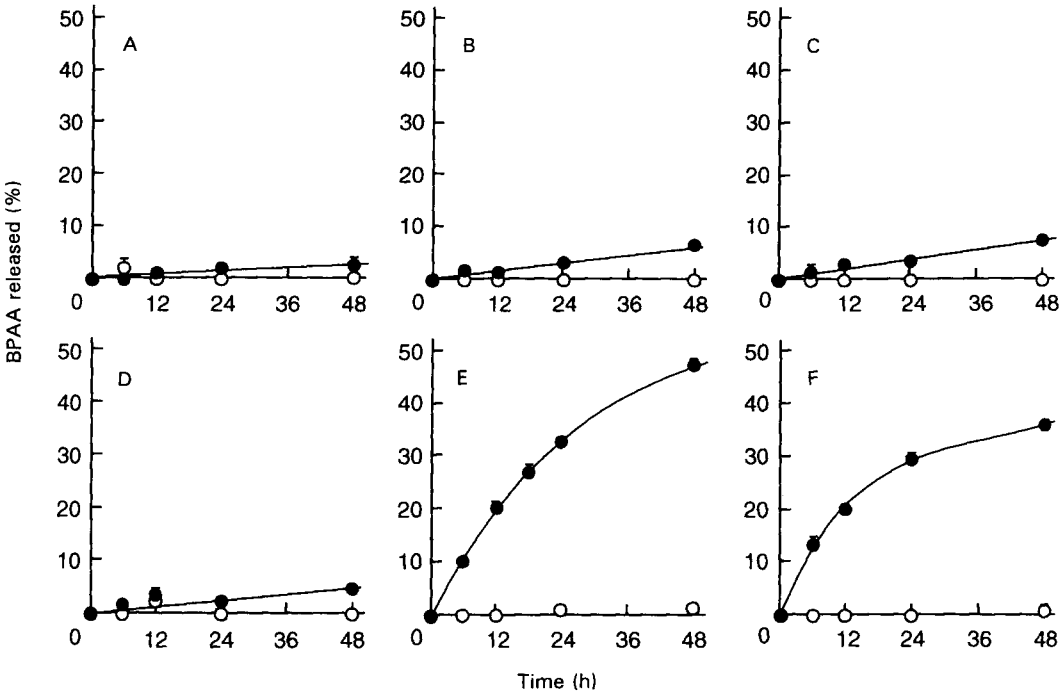

FIG. 4. Release profiles of BPAA during incubation of amide (○) and ester (●) conjugates ($1{\cdot}0 \times 10^{-5}$ M) in rat GI tract contents and in intestine homogenates in isotonic buffer/DMF (1·0 v/v) solutions at 37°C. A. stomach contents (10 w/v, pH-meter reading after the reaction (48 h) = 4·3); B. proximal small intestinal contents (10 w/v, pH = 7·2); C. distal small intestinal contents (10 w/v, pH = 7·2); D. small intestine homogenates (10 w/v, pH = 7·0); E. caecal contents (6·7 w/v, pH = 7·0); F. colonic contents (10 w/v, pH = 7·2). Each point represents the mean ± s.e. of three experiments.

other hand, the ester conjugate released the drug significantly in the caecal and colonic contents, and the rate was much higher than that to be expected to result from spontaneous hydrolysis. The anaerobic conditions accelerated the release rate of the drug in the caecal and colonic contents only slightly as compared with the aerobic conditions; the released amounts of BPAA in the 6·7 w/v caecum after 48 h were 46 and 49% under aerobic and anaerobic conditions, respectively. In the rat blood and liver homogenates, the ester conjugate released small amounts (<8%) of the drug, and the amide conjugate released no drug for 48 h (data not shown). Fig. 5 shows the release profiles of BPAA from the ester conjugate in a suspension containing various amounts of caecal contents. The release rate accelerated as the amount of the contents increased, and about 46% of the total BPAA was released in 6·7% caecal contents over 48 h. These results clearly indicate that the ester conjugate releases the drug predominantly in the rat caecum and colon. β-CyD is known to be poorly hydrolysed in the rat and human intestine, whereas it is fermented to small saccharides in the colonic microflora within 60 h (Suzuki & Sato 1985). Therefore, some fraction of the drug may be released after the ring-opening of β-CyD, although the hydrolysis mechanism (Hirayama et al 1993) should be further studied.

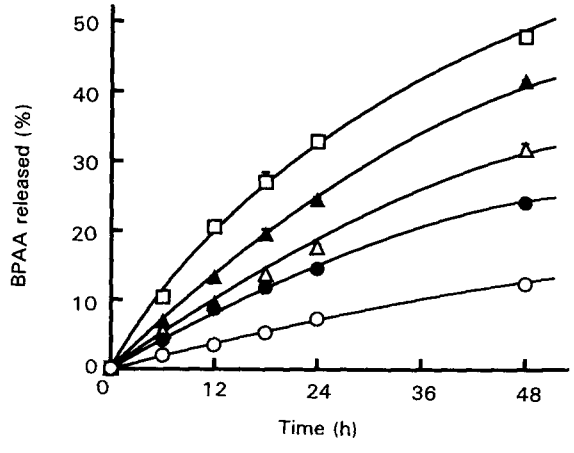

FIG. 5. Release profiles of BPAA during incubation of ester conjugate ($1{\cdot}0 \times 10^{-5}$ M) in isotonic phosphate buffer/DMF (1·0 v/v) solutions containing various amounts of rat caecal contents at 37°C. ○ without contents (pH-meter reading after the reaction (48 h) = 7·4), ● 1 w/v contents (pH = 7·1), △ 2 w/v contents (pH = 7·0), ▲ 4 w/v contents (pH = 7·0), □ 6·7 w/v contents (pH = 7·0). Each point represents the mean ± s.e. of three experiments.

The present data suggest that CyDs can function as colon-targeting carriers by conjugating covalently with drugs. This strategy may be particularly useful for drugs such as glucocorticoids, 5-aminosalicylic acid and anti-cancer drugs, for all of which selective delivery is desirable to colonic tissue to minimize systemic side effects due after absorption. These studies are in progress, together with the preparation of α- and γ-CyD conjugates of BPAA.

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